Intrinsic Defects in the T-Cell Lineage Results in Natural Killer T-Cell Deficiency and the Development of Diabetes in the Nonobese Diabetic Mouse

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T-cell–mediated autoimmune diabetes in nonobese diabetic (NOD) mice is closely associated with natural killer T (NKT)-cell deficiency. To determine whether intrinsic defects of the T-cell lineage contribute to the pathogenesis of the disease and NKT cell deficiency, we reconstituted the T-cell compartment in NOD.*scid* or BALB.*scid* mice with T-cells from NOD, nonobese diabetes-resistant (NOR), or AKR thymic precursor cells and examined the development of the NKT cell population. NKT cells developed well from AKR thymic precursor cells but not from other precursor cells in both recipient strains. Insulitis and diabetes developed only in the NOD.*scid* recipients of NOD or NOR precursor cells. When thymic precursor cells of β2-microglobulin gene–deficient AKR mice, which have a deficient NKT population, were introduced into NOD.*scid* recipients, both $CD4^+$ and $CD8^+$ T-cell populations developed and the recipient mice developed insulitis and diabetes. We conclude that NKT cells originate from a T-cell–committed thymic precursor population and that the deficiency in the NKT cell population in NOD mice results from intrinsic defects within the T-cell lineage and plays a major role in the development of autoimmune diabetes in the presence of both the NOD thymus and antigen-presenting cells. *Diabetes* 50:2691–2699, 2001

The development of type 1 diabetes in nonobese diabetic (NOD) mice results from the destruction of pancreatic β-cells by a complicated and chronic pathogenic process of islet-specific autoimmune reactions. Cumulative evidence indicates that T-cells play a major role in the pathogenesis of autoimmune diabetes in NOD mice (1–5), although other immunocytes, such as macrophages, dendritic cells, and B-cells, clearly are involved in the complicated pathogenic process (5–9). Multiple deficiencies in T-cell functions have been identified in NOD mice (10,11). However, it is not clear whether these functional deficiencies result from the developmental defects in NOD thymic education or intrinsic defects within the NOD T-cell lineage. As well, the roles of the functional deficiencies of the T-cell populations in the autoimmune pathogenic process are not well understood. If intrinsic defects within the T-cell lineage play a critical role in the pathogenesis of autoimmune diabetes in the NOD mouse, then it may be possible to correct the functional deficiencies and prevent the development of autoimmune diabetes by replacement of the T-cell lineage of the NOD mouse with that of diabetes-resistant mice.

The deficiency of the regulatory T-cell populations has long been suggested as one of the major pathogenic mechanisms of type 1 diabetes in NOD mice (12–15). A small T-cell population, the natural killer T (NKT)-cell population, was recently found to be deficient in NOD mice (16–19). A similar deficiency of the NKT cell population was also identified in human type 1 diabetic patients (20). NKT cells are a small T-cell population expressing the NK cell markers but are considered to be a major regulatory T-cell population because they produce a large amount γ-interferon (IFN-γ) and interleukin-4 (IL-4) in response to primary T-cell receptor (TCR) ligation (21,22) and are suggested to play a critical role in potentiating both the Th1 and Th2 immune responses under certain circumstances (23–26). Although the proposed mechanisms by which NKT cells are involved in immune regulation remain controversial (25,26), increasing the number of NKT cells, either by adoptive transfer of enriched NKT cells (27) or transgenic expression of the invariant TCR Vα chain dominantly used by NKT cells (28), resulted in a decrease in insulitis and diabetes in NOD mice. These studies provide strong evidence that an NKT cell deficiency contributes to the disease susceptibility. However, the mechanism that result in this NKT cell deficiency in NOD mice remains unknown.

We have developed a unique animal model to determine the origin of the NKT cell deficiency and its role in the pathogenesis of autoimmune diabetes in NOD mice. We reconstituted the T-cell compartment in NOD.*scid* or BALB.*scid* recipient mice with T-cells originating from diabetes-prone NOD mice or one of two genetically distinct diabetes-resistant strains, nonobese diabetes-resistant (NOR) or AKR mice, by introducing T-cell–committed thymic precursor cells into the thymus of neonatal recipient mice. We found that regardless of the thymic microenvironments of the recipient mice, the NKT cell population developed well only from AKR thymic precursor cells, whereas a deficient NKT cell population was found in the recipients of NOD or NOR thymic precursor

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FACS, fluorescence-activated cell sorter; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; TCR, T-cell receptor.

cells. The NOD.*scid* recipients of NOD or NOR precursor cells developed insulitis and diabetes, whereas the NOD-.*scid* recipients of AKR thymic precursor cells were free of insulitis. However, a deficient NKT cell population developed in the NOD.*scid* recipients of β2-microglobulin (β2 m) gene–deficient AKR thymic precursor cells, and these recipient mice developed insulitis and diabetes to the same degree as NOD.*scid* recipients of NOD thymic precursor cells. In contrast, none of the BALB.*scid* recipients of NOD thymic precursor cells developed insulitis. We now report that intrinsic defects within the T-cell lineage result in the deficiency of the NKT cell population, which plays a critical role in the development of autoimmune diabetes in the presence of both the NOD thymus and antigen-presenting cells.

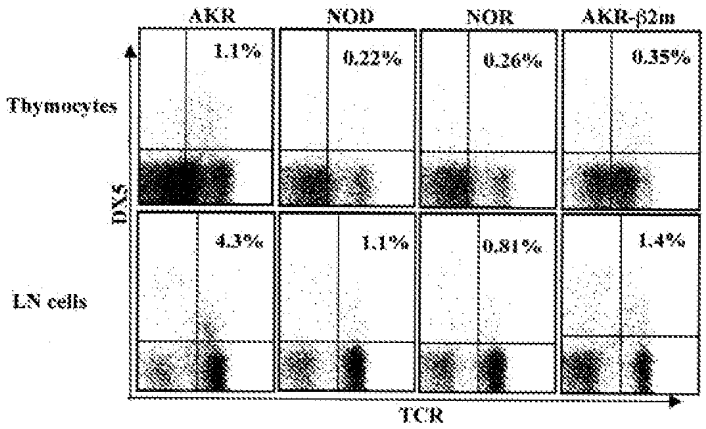

FIG. 1. Deficient NKT cell population in NOD, NOR, and AKR-β2 m mice. Thymocytes and lymph node cells were isolated from NOD, NOR, and AKR-β2 m mice, stained with anti-TCRβ chain and DX5 antibodies, and analyzed by FACS. As a control, cells of AKR mice were also analyzed. In AKR mice, the DX5$^+$TCR$^+$ NKT cell population was clearly identified in the thymocytes and lymph node cells, but identical populations in the thymus and lymph nodes of NOD, NOR, and AKR-β2 m mice were deficient. Data represent the results from at least five mice per group.

RESEARCH DESIGN AND METHODS

Mice and antibodies. All mice used in this study were purchased from the Jackson Laboratory (Bar Harbor, ME), and all antibodies were purchased from Pharmingen Canada (Mississauga, Ontario, Canada).

Isolation of CD25$^+$CD44$^-$ thymic precursor cell population and injection of purified precursor cells into the thymus of neonatal NOD.*scid* recipients. Thymocytes from 4-week-old donor mice were incubated with antibodies to CD4 (RM4-4), CD8 (53.6.7), and CD3 (145.2C11), washed, and then incubated with baby rabbit complement. Enriched triple-negative thymocytes were then stained with FITC-labeled antibodies to CD4, CD8, CD44, TCRβ, and PE (R-phycoerythrin)-labeled antibodies to CD25. The CD25$^+$CD44$^-$ thymic precursor cells were purified by cell sorting. The purified CD25$^+$CD44$^-$ cells (>95% pure) were suspended in a phosphate-buffered saline (PBS) solution at a concentration of 2.5×10^8 cells/ml. Two- to 3-day-old NOD.*scid* mice were used as recipients of thymic precursor cells. Intrathymic injection was performed as described previously (29). Briefly, the recipient mice were anesthetized and immobilized, and a midline upper thoracic incision was made followed by a "V" excision of the upper sternum/ribcage under a stereomicroscope. Purified CD25$^-$CD44$^+$ cells (0.5×10^6 cells/mouse, resuspended in 2 μl of PBS) were injected into both lobes of the thymus of each recipient under the stereomicroscope. As controls, littermates of the recipients received an injection of PBS solution alone.

Examination of T-cell reconstitution in NOD.*scid* recipients. Four weeks after injection of the precursor cells, T-cell reconstitution of the recipient mice was monitored weekly by fluorescence-activated cell sorter (FACS) analysis of peripheral blood mononuclear cells (PBMCs). PBMCs were stained with antibodies to TCRβ, Thy1.1, Thy1.2, CD4, CD8, and B220 (Pharmingen Canada). Lymph node cells and splenocytes of the NOD.*scid* recipient mice were also examined when the mice were killed. A small number of Thy1.2$^+$ T-cells were detected in two recipients of AKR thymic precursor cells. These cells were likely of host origin that developed as a result of a "leaky" *scid* mutation. These mice were removed from the study. The expression of TCR Vβ chains in the thymocytes of recipient mice was examined by reverse transcriptase–polymerase chain reaction using a common primer for the C fragment and 17 primers specific for individual Vβ chains (30).

Reconstitution of B-cells. B-cells were isolated from splenocytes of 5- to 8-week-old NOD mice using B220 antibody–coated beads and a Mini MACS system (Miltenyi Biotec, Auburn, CA). The purity of B220$^+$ cells was higher than 95%. The B-cells were resuspended in PBS (1×10^8 cells/ml) and transferred into recipient mice by intraperitoneal injection.

Assay for cell proliferation and cytokine production. In mixed lymphocyte reactions, 1×10^6 responder splenocytes were mixed with 0.5×10^6 irradiated stimulator cells and incubated for 3 days and pulsed for an additional 16 h with [^{3}H]thymidine. To measure NKT cell function, the NOD.*scid* recipient mice received an injection of antibody to CD3 (10 μg/mouse i.p.). One hour after injection, spleens were removed and splenocytes were resuspended at 5×10^6 cells/ml. The culture supernatant was collected at 2 and 4 h, and the concentration of IFN-γ and IL-4 in the supernatant was determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Measurement of blood glucose. Blood glucose levels of nonfasting mice were measured as described elsewhere (31). The mean blood glucose level of nonfasting AKR mice was 7 mmol/l. In this study, nonfasting animals with blood glucose levels greater than 16 mmol/l for 3 consecutive days were scored as diabetic.

Detection of NKT cells. Lymph node cells from NOD.*scid* or BALB.*scid* recipients were preincubated with antibody against CD16/32 (2.4G2; Pharmingen Canada) in PBS containing 2% fetal calf serum. The cells were then incubated with biotinylated DX5 antibody on ice for 20 min. Control cells were incubated with dilution buffer. After washes, cells were incubated with streptavidin-perCP and anti–TCR-β–FITC antibody. The cells were resuspended and analyzed by FACS.

RESULTS

NOD and NOR mice possess deficient NKT cell populations in the thymus and periphery. Diabetes-resistant NOR mice share an identical major histocompatibility complex (MHC) haplotype and most of the genome with NOD mice, and NOR mice may possess many defects in their immune system similar to those in NOD mice, including defective thymic education. To determine whether NOR mice develop an NKT cell deficiency as do NOD mice, we examined the DX5$^+$TCR$^+$ cell population in the thymus and periphery of NOR and NOD mice, because DX5$^+$TCR$^+$ cells represent the majority of NKT cells in NK1.1-negative strains (Y.Y., B.M., J.-W.Y., manuscript submitted). The DX5$^+$TCR$^+$ cell population in NOR and NOD mice was compared with that in AKR mice (Fig. 1) because the NKT cell population develops well in AKR mice. We found very few DX5$^+$ T-cells in the thymus and periphery of NOR and NOD mice. In fact, the NKT cell population in NOD and NOR mice was similar to that found in AKR mice with a β2 m gene deficiency (AKR-β2 m mice; Fig. 1). NKT cell development in AKR-β2 m mice was defective because of a failure of expression of the CD1/β2 m complex. Analysis of the DX5$^+$TCR$^+$ cell population in various organs clearly showed that the NKT cell population is deficient in both NOD and NOR mice.

The T-cell compartment of NOD.*scid* mice can be reconstituted with thymic precursor cells isolated from diabetes-prone and diabetes-resistant mice. To determine whether the NKT cell deficiency in NOD and NOR mice is associated with the thymic microenvironment, we reconstituted the T-cell compartment of NOD-.*scid* mice with thymic precursor cells isolated from NOD, NOR, and AKR mice (Fig. 2*A*). The purified thymic precur-

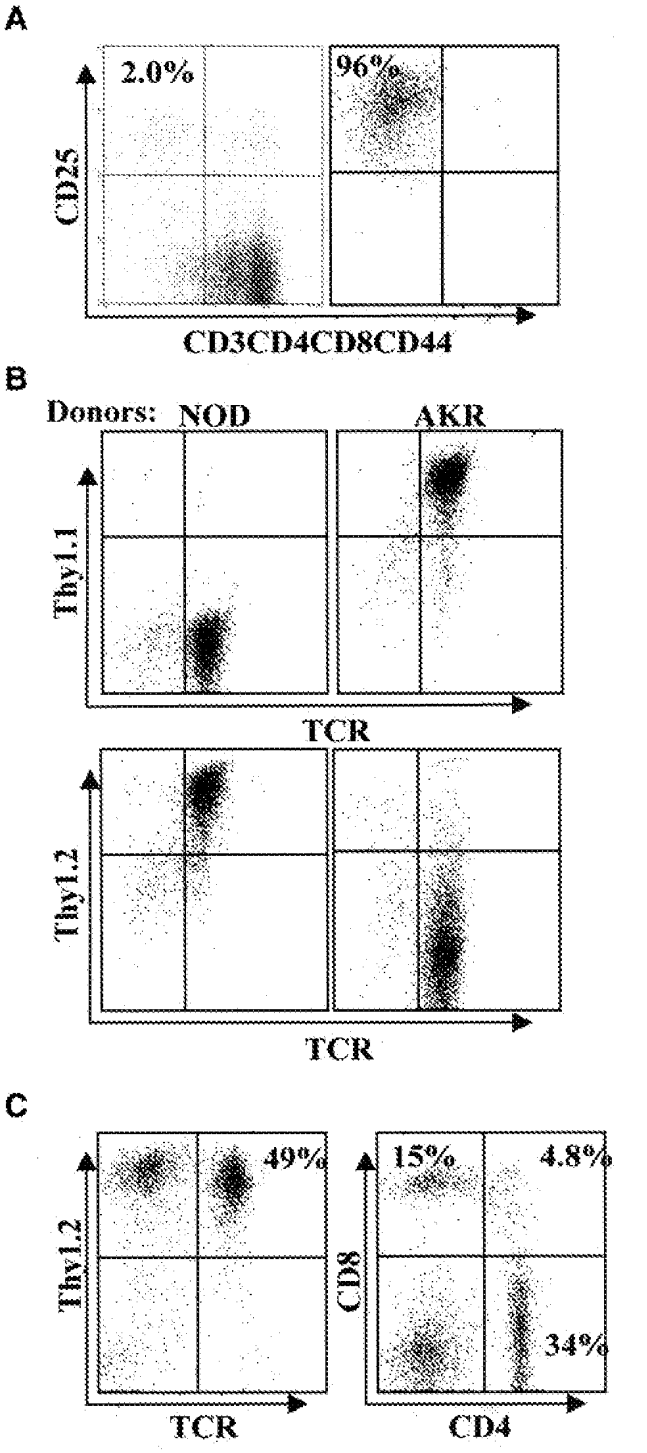

FIG. 2. Reconstitution of the T-cell compartment in the NOD.*scid* mice with NOD, NOR, and AKR precursor cells. *A*: In young donor NOD, NOR, or AKR mice, <2% of the thymocytes were $CD25^+CD44^-$ cells. These cells were enriched and purified from NOD, NOR, and AKR donors. The purity of the thymic precursor cells isolated from donor mice was ~95%. *B*: More than 80% of lymph node cells of the reconstituted NOD.*scid* mice were T-cells. In the recipients of NOD precursor cells, all of the T-cells were Thy1.2 positive, whereas all of the T-cells in the recipients of AKR precursor cells were Thy1.1 positive but Thy1.2 negative. Lymph node cells from the recipients of NOR precursor cells were identical to those in the recipients of NOD precursor cells. *C*: A large portion of the thymocytes in the reconstituted NOD.*scid* mice expressed a high level of TCR. However, there was only a small population of CD4 and CD8 double-positive thymocytes in these recipient mice.

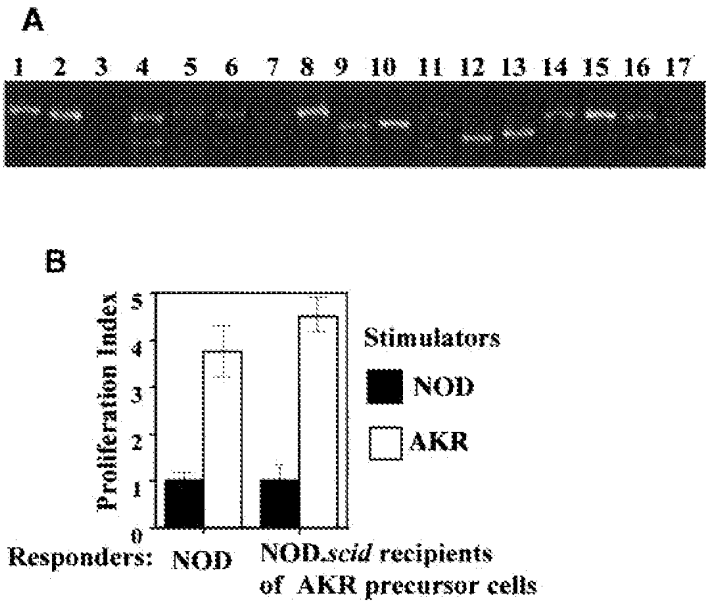

FIG. 3. TCR repertoire and functions of the T-cells of NOD.*scid* recipients. *A*: Reverse transcriptase–polymerase chain reaction analysis showed that a diversified TCR repertoire developed in the NOD.*scid* recipient mice of AKR precursor cells. The expression of Vβ3 and Vβ17 chains was not detected. Similar patterns of TCR Vβ usage were seen in the recipients of NOD and NOR precursor cells. *B*: Lymphocytes from NOD.*scid* recipients of AKR precursor cells (responder) proliferated against irradiated AKR splenocytes (stimulator), but these cells did not respond to irradiated NOD splenocytes. The proliferation response of lymphocytes from the recipients of AKR precursor cells was very similar to the response of NOD lymphocytes (responder). Bars indicate ranges.

sor cells were injected into the thymus of NOD.*scid* neonates at 3 days of age. Thymic precursor cells isolated from syngeneic and allogeneic donor mice developed equally well into mature T-cells in these NOD.*scid* recipients. T-cell populations were detected in the circulation of the recipients within 3–4 weeks after thymic injection. When the lymph node cells were analyzed, it was found that >80% of the lymph node cells in the reconstituted NOD.*scid* mice were T-cells (Fig. 2*B*). T-cells in the recipients of NOD and NOR thymic precursor cells expressed Thy1.2 antigen, whereas all of T-cells in the recipients of AKR thymic precursor cells expressed Thy1.1 antigen (Fig. 2B). No T-cells were detected in control NOD.*scid* mice. These results show that all T-cells in the recipients originated from donor precursor cells.

The spleens of the reconstituted mice contained a small proportion (5–20%) of T-cells, and most of the thymocytes were CD4 or CD8 single-positive cells; only a small number (<5%) were $CD4^+CD8^+$ double-positive cells (Fig. 2*C*), indicating that all of the donor thymic precursor cells developed rapidly into mature thymocytes in the recipients. The donor-derived T-cell populations remained for >24 weeks in the NOD.*scid* recipients. B-cells were virtually undetectable in these recipient mice.

We then analyzed the TCR Vβ chain repertoire in the reconstituted NOD.*scid* mice and found a diversified TCR Vβ chain usage in both the splenocytes and thymocytes of all reconstituted mice (Fig. 3*a*). As was previously found in NOD mice (32), both Vβ3- and Vβ17-expressing T-cells were missing in all of the NOD.*scid* recipients, indicating that the TCR repertoire of the NOD.*scid* recipients was

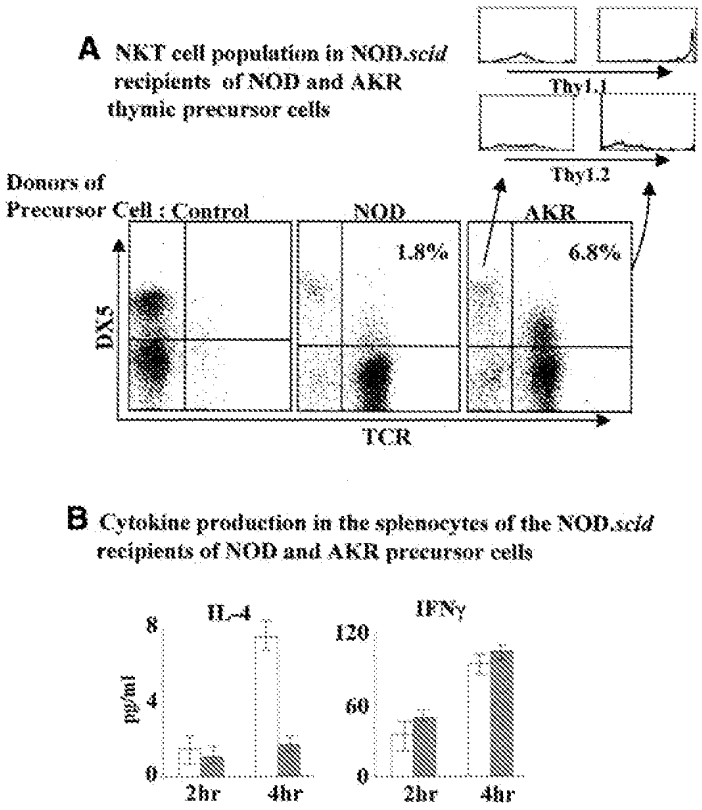

FIG. 4. Development of NKT cell population in NOD.*scid* recipients. *A*: $DX5^+$ NK cells but not T-cells were found in lymph node cells of control NOD.*scid* mice. In the NOD.*scid* recipients of NOD precursor cells, 78% of lymph node cells were T-cells with <2% $DX5^+$ T-cells. Identical results were obtained from the recipients of NOR precursor cells. In contrast, a far greater number of $DX5^+TCR^+$ cells were found in the lymph node cells of NOD.*scid* recipients of AKR precursor cells. $DX5^+TCR^+$ NKT cells expressed Thy1.1 antigen; these cells were of AKR origin. $DX5^+TCR^-$ NK cells were Thy1.1-negative, with a low level of expression of Thy1.2 antigen; these cells were of NOD.*scid* host origin. *B*: IFN-γ and IL-4 production of splenocytes from NOD.*scid* recipients of NOD and AKR precursor cells. Splenocytes of the recipients of AKR precursor cells (□) produced more IL-4 ($P < 0.001$) and a similar amount of IFN-γ compared with the recipients of NOD precursor cells (■) within 4 h of culture after the injection of antibody to CD3 antigen. Data represent the results from four mice per group.

shaped by NOD thymic education. Furthermore, the lymphocytes from NOD.*scid* recipients of AKR thymic precursor cells responded to irradiated splenocytes of AKR mice in a mixed lymphocyte reaction but not to NOD splenocytes (Fig. 3*B*). This result indicates that the mature T-cell populations that developed in the NOD.*scid* recipients were restricted by the host MHC expressed in the thymus.

NKT cell development differs among NOD.*scid* recipients of NOD, NOR, and AKR thymic precursor cells. We next compared the development of the $DX5^+$ NKT cell population among the NOD.*scid* recipients of NOD, NOR, and AKR thymic precursor cells. Very few $DX5^+$ NKT cells were found in the lymph node cells of NOD.*scid* recipients of NOD or NOR thymic precursor cells. In contrast, a significant number of $DX5^+$ NKT cells were detected in the NOD.*scid* recipients of AKR thymic precursor cells (Fig. 4*A*). In these mice, the $DX5^+$ NKT cells expressed Thy1.1 antigen but not Thy1.2, indicating that the $DX5^+$ NKT cells were of AKR donor origin. In contrast, $DX5^+$ NK cells did not express Thy1.1, but some of these cells expressed a low level of Thy1.2, indicating that the $DX5^+TCR^-$ NK cells were derived from the NOD.*scid* host.

Cytokines produced by NKT cells favor the Th2 immune response in TCR-mediated primary activation (21,22,33). To determine whether the NKT cell cytokine profiles differ among the reconstituted NOD.*scid* mice, we injected antibody to CD3 antigen into the NOD.*scid* recipients of NOD or AKR precursor cells and measured the production of IL-4 and IFN-γ in the splenocytes. Within 4 h of antibody injection, splenocytes from the recipients of AKR precursor cells produced a much greater amount of IL-4 ($P < 0.001$) and a similar amount of IFN-γ compared with the splenocytes from the recipients of NOD precursor cells (Fig. 4*B*). This result indicates that the NOD T-cell populations differ from the AKR T-cell populations in the ability to release IL-4 quickly after activation. Because the stimulation protocol used in these experiments largely targets NKT cells (21), this difference is likely to be due to the defective development of NKT cells from NOD thymic precursor cells.

Deficient NKT cell population correlated with the development of autoimmune diabetes in the reconstituted NOD.*scid* mice. To determine whether the reconstituted NOD.*scid* mice would develop insulitis and diabetes, we transferred purified B-cells from young NOD mice (4–5 weeks) into these recipient mice (4×10^7/recipient), because B-cells are required for the development of T-cell–mediated autoimmunity in NOD mice (7,8,34,35). The transfer of NOD B-cells alone did not induce insulitis and diabetes in control NOD.*scid* mice. However, mild to severe insulitis developed in 10 of 12 NOD.*scid* recipients of NOD thymic precursor cells along with NOD B-cells (Tables 1 and 2), and five of these mice developed diabetes within 12 weeks. The total number of T-cells in the NOD.*scid* recipients was <20% (8×10^6/mouse) of that found in normal NOD mice, and a very few transferred B-cells remained in these recipients, likely as a result of $CD8^+$ T-cell–mediated rejection (35). The low number of T- and B-cells in NOD.*scid* recipients probably resulted in the relatively low incidence of overt diabetes. Mild to severe insulitis also developed in all nine NOD.*scid* recipients of NOR thymic precursor cells, and three of these NOD.*scid* recipients developed diabetes. However, insulitis was not detected in any of the eight NOD.*scid*

TABLE 1
The development of autoimmune diabetes in reconstituted NOD.*scid* mice*

Donor of precursor T-cells	Incidence of insulitis†	Incidence of diabetes
NOD	10/12 (83)	5/12 (42)
NOR	9/9 (100)	3/9 (33)
AKR	0/8 (0)	0/8 (0)
β2 m–deficient AKR	10/10 (100)	6/10 (60)

Data are *n* (%). *Urine and blood glucose levels of NOD.*scid* recipient mice of thymic precursor cells from various donors were monitored twice a week. The incidence of diabetes was examined at 20 weeks after adoptive transfer of purified NOD B-cells into different groups of reconstituted NOD.*scid* mice. †At the termination of each experiment, all mice were killed for histological examination of the pancreatic islets. At least 20 islets from each mouse were examined. Any mouse that showed lymphocytic infiltration in >20% of the examined islets was considered to be positive for insulitis.

TABLE 2
Histological examination of insulitis in reconstituted NOD.*scid* mice*

Donor of precursor T-cells	Insulitis grade (%)†			
	0	1	2	3
NOD	8.3	11.6	26.6	53.3
NOR	11.6	18.3	26.6	43.3
AKR	97.2	2.8	0	0
β2 m–deficient AKR	14.3	25.7	35.7	24.3

*Insulitis was analyzed in nondiabetic NOD.*scid* recipient mice of thymic precursor cells from various donors at 20 weeks after the adoptive transfer of NOD B-cells. Diabetic NOD.*scid* recipients were not included in this table, as most of the islets examined were atrophied. †Between 60 and 70 islets from three nondiabetic mice (at least 20 islets/mouse/group) were examined. 0, normal; 1, infiltration in <25% of the islet; 2, 25–50% infiltration of the islet; 3, >50% infiltration of the islet.

recipient mice of AKR thymic precursor cells at 25 weeks of age in the presence of NOD B-cells (Tables 1 and 2).

Thus, insulitis and diabetes developed in almost all of the NOD.*scid* mice reconstituted with NOD and NOR thymic precursor cells, and these mice developed deficient NKT cell populations. In contrast, NOD.*scid* mice reconstituted with AKR thymic precursor cells contained a normal NKT cell population and were resistant to the development of insulitis and diabetes. To determine whether the resistance to insulitis and diabetes in the NOD.*scid* mice reconstituted with T-cells of AKR origin was associated with the development of the NKT cell population, we further reconstituted NOD.*scid* mice with thymic precursor cells from β2 m gene–deficient AKR mice. NKT cells are positively selected by CD1 antigen expressed on double-positive thymocytes (36), but the double-positive thymocytes of β2 m gene–deficient AKR mice do not express CD1 antigen (Fig. 5*A*). Therefore, the NKT cell development in these β2 m gene–deficient AKR mice was defective (Fig. 1). In addition, the $CD8^+$ T-cell population did not develop in these β2 m gene–deficient AKR mice because of lack of the expression of class I MHC molecules (Fig. 5*B*). However, when thymic precursor cells from β2 m gene–deficient AKR mice were introduced into NOD.*scid* recipients, both $CD8^+$ and $CD4^+$ T-cell populations developed (Fig. 5*B*). The restoration of the $CD8^+$ T-cell population was evidently due to positive selection by host MHC class I molecules. However, the NKT cell population was deficient in these recipient mice (Fig. 5*C*) because the $CD4^+CD8^+$ double-positive thymocytes were of donor origin, and these cells were unable to express CD1d for positive selection of NKT cells. In addition, these mice did not produce detectable IFN-γ or IL-4 after anti-CD3 antibody injection (data not shown). Examination of the pancreatic islets of these recipient mice revealed that all mice had developed insulitis (Table 2) and 6 of 10 had developed diabetes (Table 1). These results indicate that T-cells of AKR origin can induce diabetes in NOD.*scid* recipients if the NKT cell population does not fully develop and that thymic precursor–derived NKT cells play a critical role in the prevention of autoimmune diabetes in the reconstituted NOD.*scid* mice.

Intrinsic defects expressed within the NOD thymic precursor cells result in a defective development of the NKT cell population, which cannot be corrected by either a BALB.*scid* thymic microenvironment or interaction with AKR thymic precursor cells. Because the NKT cell population in the reconstituted NOD.*scid* mice developed from donor thymic precursor cells, we asked whether the thymic precursor cells express DX5 antigen. We enriched $CD25^+$ thymocytes from AKR mice and found that $CD25^+$ thymocytes did not express DX5 antigen and DX5 antigen–expressing cells were present only in $CD25^-$ thymocytes (Fig. 6*A*). We then examined the expression of CD1d antigen in NOD and AKR mice and found that the expression levels of CD1d antigen in

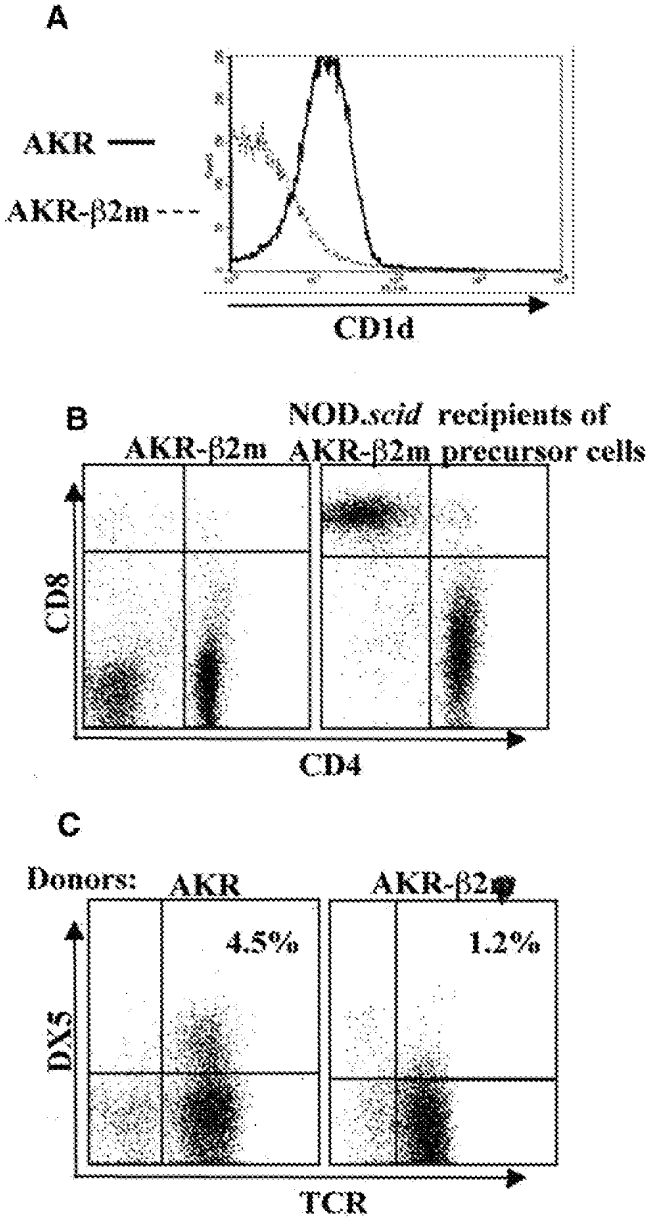

FIG. 5. NKT and T-cell populations derived from thymic precursor cells of β2 m gene–deficient AKR mice. *A*: $CD4^+CD8^+$ double-positive thymocytes of β2 m gene–deficient AKR mice did not express CD1d antigen. *B*: The $CD8^+$ T-cell population was missing in β2 m gene–deficient AKR mice, but the $CD8^+$ T-cell population was restored when thymic precursor cells from β2 m gene–deficient AKR donors were introduced into NOD.*scid* recipient mice. *C*: $DX5^+$ NKT cells were deficient in the NOD.*scid* recipients of thymic precursor cells of β2 m gene–deficient AKR mice, but an identical population in the NOD.*scid* recipients of AKR thymic precursor cells developed well. Data represent the results from more than three mice per group.

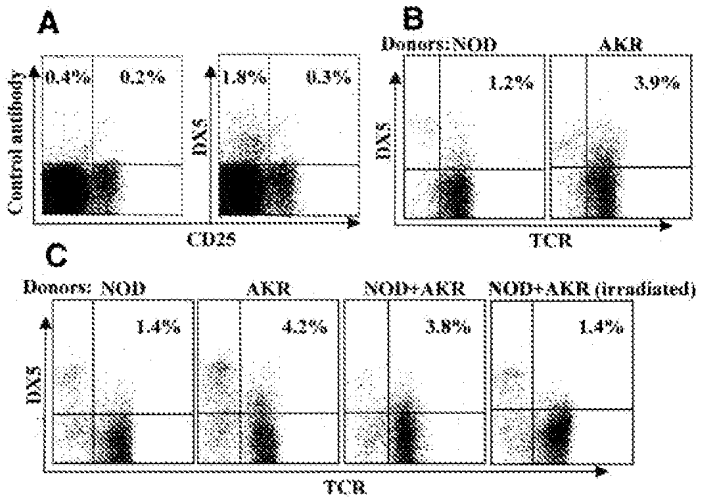

FIG. 6. NKT cell differentiation from CD25$^+$ thymocytes. *A*: In thymocytes of AKR mice, DX5$^+$ cells were present in only CD25$^-$ thymocytes. CD25 and DX5 double-positive thymocytes were virtually undetectable. Data represent the results from more than four mice per group. *B*: NKT cell population developed in BALB.*scid* recipients of NOD thymic precursor cells and AKR thymic precursor cells. In the lymph nodes of recipient mice of NOD precursor cells, ~1% of cells were DX5$^+$TCR$^+$, whereas in BALB.*scid* recipients of AKR precursor cells, >3% of lymph node cells were DX5$^+$TCR$^+$. Data represent the results from three mice per group. *C*: NKT cell population developed well in the NOD.*scid* recipients of the thymic precursor cells from AKR (4.2%) or NOD and AKR (mixed in 1:1 ratio) (3.8%) but not in the recipients of the thymic precursor cells of NOD + irradiated AKR (1.4%) or NOD donors (1.4%). Data represent the results from three mice per group.

CD25$^+$CD44$^-$ and CD4$^+$CD8$^+$ thymocytes, as well as in B-cells, were identical between the two strains (data not shown). This result indicates that the DX5-expressing NKT cells differentiated from late-stage premature T-cells after the injection of CD25$^+$CD44$^-$ thymic precursor cells into the thymus of recipient mice and that the defective development of the NKT cell population in NOD mice is not due to a low level of CD1d expression.

To determine whether a different thymic microenvironment has any effect on the development of the NKT cell population, we injected AKR or NOD thymic precursor cells (CD25$^+$CD44$^-$) into the thymus of neonatal BALB-*.scid* mice and examined the development of the NKT cell population in the adult recipients. The numbers of NKT cells were very low in the BALB.*scid* recipients of NOD precursor cells. However, a well-developed NKT cell population was found in the recipients of AKR precursor cells (Fig. 6*B*). These results were similar to those obtained from the reconstituted NOD.*scid* mice. Therefore, defective development of the NKT cell population in these reconstituted mice resulted from the thymic precursor cells but not from the thymic microenvironment.

We next tested whether the defective differentiation of NOD thymic precursor cells can be corrected in the presence of AKR precursor cells by introducing mixed (1:1 ratio) NOD and AKR thymic precursor cells into NOD.*scid* recipients. T-cells of both AKR and NOD origin developed in these recipient mice, and there was a moderate increase in the number of NKT cells as compared with the number in recipients of NOD precursor cells alone. However, when the thymic precursor cells of AKR donors were irradiated and then mixed with NOD thymic precursor cells, only T-cells of NOD origin developed, and the NKT cell population was deficient (Fig. 6*C*). We also injected NOD thymic precursor cells mixed with irradiated CD4$^+$CD8$^+$ AKR thymocytes (1:2) into NOD.*scid* recipients and found only T-cells of NOD origin with a deficient NKT cell population (data not shown). Thus, cell-cell interaction with AKR thymocytes could not rescue the defective development of NKT cells from NOD thymic precursor cells.

DISCUSSION

A triple-negative thymocyte population (CD25$^+$CD44$^-$, CD117$^-$, and CD3$^-$CD4$^-$CD8$^-$) has been identified as the earliest precursor cell type irreversibly committed to the T-cell lineage; these cells do not develop into any other cells of hematopoietic origin (37–40). If CD25$^+$CD44$^-$ thymocytes are isolated from a desired donor and introduced into the thymus of NOD.*scid* recipient mice, then not only should T-cell populations developed in the recipient mice be of donor origin, but more importantly, the thymic education of the T-cell populations should be determined entirely by the thymic microenvironment of the recipient. Consequently, the mature T-cells will be restricted by the recipient's MHC, and their immune response will be carried out in conjunction with the antigen-MHC complex of the recipient's antigen-presenting cells. On the basis of this concept, we have developed a unique animal model in which the genetic background of the T-cell populations can be different from that of all of the other cell populations in the animal.

The analysis of reconstituted NOD.*scid* mice showed that both syngeneic and allogeneic CD25$^+$CD44$^-$ thymic precursor cells developed equally well into mature T-cell populations under an identical NOD thymic education. More importantly, the development of T-cell populations in the recipient mice was shaped by NOD thymic education. As a consequence of the host thymic education, the T-cells of the NOD.*scid* recipients, regardless of donor genetic background, recognized the host MHC but not the donor MHC as self. Therefore, using our unique animal model would allow us to distinguish the different pathogenic roles of thymic education and intrinsic defects within the T-cell lineage of NOD mice when T-cells of different origins were educated by an identical thymic microenvironment.

To determine whether the defective thymic education contributes to the NKT cell deficiency in NOD mice, we first examined the development of NKT cells in NOR mice, because NOR mice share an identical MHC locus and most of the genome with NOD mice. We found that NKT cell development is defective in both NOD and NOR mice. Because most NKT cells develop from the thymus, the defective development of the NKT cell population in NOD and NOR mice may be due to their MHC or other defective factors in their thymic microenvironment. It has been shown that IL-7, which is a product of thymic stromal cells, is required for the development and maturation of NKT cells (41,42). Exogenous IL-7 improved the IL-4 production of NOD thymocytes (43), suggesting defective NOD stromal cell functions.

To determine the origin of the NKT cell deficiency in NOD and NOR mice, we reconstituted both NOD.*scid* and BALB.*scid* mice with thymic precursor cells from NOD, NOR, and AKR donors. If the NKT cell deficiency is a result

of defects in NOD thymic education, then the reconstituted NOD.*scid* mice would develop deficient NKT cells, whereas the BALB.*scid* recipient mice would have normal NKT cell development regardless of the origin of thymic precursor cells. In contrast, if intrinsic defects within the T-cell lineage of NOD mice result in the NKT cell deficiency, then this deficiency will be associated only with T-cells of NOD origin. We found that a normal NKT cell population developed from AKR thymic precursor cells. In contrast, a deficient NKT cell population developed from NOD and NOR thymic precursor cells in both NOD.*scid* and BALB.*scid* mice. These results show that the genetic defects in the thymic precursor cells of NOD and NOR mice are the major cause of the NKT cell deficiency, whereas the thymic microenvironment of NOD mice does not block NKT cell development.

We further asked whether the NKT cell deficiency is due to the defective expression of CD1 and other selecting factors on the surface of NOD thymocytes, because it has been shown that NKT cells are positively selected by CD1/β2 m complex expressed on $CD4^+CD8^+$ thymocytes (44). However, we found that the expression level of CD1d on $CD4^+CD8^+$ thymocytes was identical in NOD and AKR mice. In addition, we found that the defective NKT cell development cannot be rescued when the NOD thymic precursor cells mixed with irradiated AKR thymic precursor cells or $CD4^+CD8^+$ thymocytes before injection into NOD.*scid* recipients. Thus, the failure in NKT cell development is unlikely to be due to defects of cell surface markers. Instead, defects in the intracellular signals required for NKT cell differentiation may be responsible for the deficiency.

Consistent with our results, both Src family tyrosine kinase Fyn and transcription factor Ets1 have been shown to be required for the development of the NKT cell population (45,46). Both Fyn and Ets1 are involved in the intracellular signal pathway for NKT cell development. Our preliminary results showed that the T-cell lineage of NOD mice expresses the Ets1 gene at a level identical to that in T-cells in AKR mice and that there is no mutation in the Ets1 gene coding frame (unpublished data). However, it was reported that polymorphism in the 3′ nontranslating region of Ets1 may be associated with autoimmune syndromes (47). The mechanisms of the NKT cell deficiency in NOD mice remain to be elucidated. However, our results provide invaluable information to facilitate identification of the gene(s) responsible for the NKT cell deficiency. In addition, the results of the current study provide direct evidence that NKT cells develop from T-cell–committed precursor cells. It is known that NKT cells have a close ontogenic relationship with the T-cell lineage (48,49), but it was unclear from which precursor cells the NKT cells differentiate. We found that NKT cells develop from $CD25^+CD44^-$ thymic precursor cells, in which a TCR gene rearrangement has taken place (38). However, these thymic precursor cells do not express the NK cell marker DX5 antigen (Fig. 6*A*). These thymocytes are precursor cells for both T and NKT cells, and our results further indicated that during development, TCR expression occurs before the expression of NK cell markers. The expression of NK cell markers may be an indicator of NKT cell maturation (48).

In NOD.*scid* recipient mice reconstituted with T-cells of different origins, a sharp contrast among groups of recipient mice was observed with respect to the development of insulitis and diabetes. The NOD.*scid* recipients of NOD and NOR thymic precursor cells developed insulitis, and some of these mice became diabetic, but none of the NOD.*scid* recipients of AKR precursor cells developed insulitis. Because the donor thymic precursor cells experienced an identical thymic education mediated by NOD MHC molecules, all of the three groups of NOD.*scid* recipient mice should contain a similarly high frequency of autoreactive T-cells against β-cell autoantigens (50–52). However, these autoreactive T-cells were unable to attack pancreatic β-cells in the NOD.*scid* recipients of AKR thymic precursor cells. This result provides direct evidence that the intrinsic defects of the NOD T-cell lineage play a critical role in the pathogenesis of autoimmune diabetes, and the replacement of the T-cell lineage of NOD mice with the one of different origin can prevent autoimmune diabetes. Conversely, the development of insulitis and diabetes in the NOD.*scid* mice reconstituted with thymic precursor cells from β2 m gene–deficient AKR mice indicated that T-cells of different origin can be diabetogenic under NOD thymic education when the regulatory factors are removed. Furthermore, our results showed the NKT cell deficiency is strongly associated with the development of autoimmunity in the reconstituted NOD.*scid* mice. The deficient NKT cell population developed from NOD and NOR thymic precursor cells in the NOD.*scid* recipients correlated with the development of autoimmune diabetes, and the resistance to diabetes in the NOD.*scid* reconstituted with AKR thymic precursor cells was abolished when the development of NKT cells was defective as a result of the β2 m gene deficiency.

Therefore, consistent with other studies (27,28), our results showed that NKT cells can suppress the development of autoreactive T-cells and that the NKT cell deficiency contributes to the pathogenesis of autoimmune diabetes in NOD mice. However, the underlying mechanisms by which NKT cells control autoimmunity are still not well understood. The defective production of both IL-4 and IFN-γ by NKT cells has been indicated as a pathogenic role (19,27). We detected a higher IL-4 production in splenocytes of NOD.*scid* mice containing T-cells of AKR origin. However, we also found that NKT cells purified from spleens of AKR mice produce a large dose of IFN-γ in culture (unpublished observation). More detailed analysis will be conducted using thymic precursor cells from IL-4 or IFN-γ gene-deficient donors to understand which cytokine is important or whether the cytokine profile is key in NKT cell–mediated immunoregulation.

NOR and NOD mice share ~88% of the genome, including the MHC locus that confers susceptibility to autoimmune diabetes (53,54). Although NOR mice are resistant to insulitis and diabetes, the NOD.*scid* recipients of NOR thymic precursor cells developed insulitis and diabetes, as well as a deficient NKT cell population. It is very likely that the T-cell lineage in NOR mice shares genetic defects with those of NOD T-cells and that NOR T-cells are diabetogenic when they are developed and function in the immune system of NOD mice. Thus, diabetes resistance in NOR mice is not directly related to the T-cell lineage but to

other immunocytes, such as antigen-presenting cells. This finding is consistent with other studies showing differences in the expression and regulation of the MHC genes and the function of antigen-presenting cells between NOD and NOR mice (54–57).

Autoimmune diabetes in NOD mice is a polygenic disease, and multiple genetic defects have been associated with its pathogenesis. However, the biological effects of the genetic defects and the cell population expressing a specific genetic defect remain largely unknown. By replacing the T-cell lineage in NOD mice with lineages from different genetic backgrounds, we were able to identify the pathogenic roles played by the intrinsic defects of T-cells in NOD mice. The results of this study will be useful for further identification of the genes involved in NKT cell development and NKT cell deficiency in NOD mice. Furthermore, the unique animal model developed for this study, in which specific cell lineages of the immune system can be made genetically distinct from each other, will be invaluable for studies on the development of the immune system and for the determination of genetic factors involved in immune regulation and dysfunction, such as those that occur in autoimmune diseases and inflammatory responses.

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